INFECTION AND IMMUNITY, Aug. 1978, p. 546-555
0019-9567/78/0021-0546$02.00/0
Copyright © 1978 American Society for Microbiology
Vol. 21, No. 2
Printed in U.S.A.

Extraction of Streptococcal Type 12 M Protein by Cyanogen Bromide

KENNETH L. VOSTI* AND W. KENT WILLIAMS

Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

Received for publication 10 April 1978

Conditions for the release of streptococcal type 12 M protein from whole cells by cyanogen bromide are described; they demonstrated that methionine is not essential to the structural arrangements which account for some of its immunological and biological properties. The released M protein was separated from other proteins by column chromatography with hydroxylapatite. The type-specific molecules which reacted with precipitating antibodies were found only in the 0.3 M eluate, formed zones with mobilities <12% of that of the dye front on electrophoresis in the standard acrylamide disc gel system, formed at least four bands in sodium dodecyl sulfate-acrylamide disc gels with molecular weights ranging from 12,000 to 23,000, and stimulated the formation of opsonic antibodies in rabbits. Cyanogen bromide provides a highly specific method for the release of M proteins which should prove particularly useful in analyses of structural-functional relationships among different M proteins.

M proteins are the most important of the various cellular antigens of group A streptococci. They confer a unique antiphagocytic property to group A streptococci and are responsible for the stimulation of type-specific, opsonic antibodies, which provide the infected or immunized host with protective immunity (10, 16). Generally M proteins have been extracted by the method of Lancefield (14), which consists of heating whole cells or their isolated cell walls at 100°C for 10 to 15 min in a solution of HCl at pH 2. Further purification of these crude acid extracts resulted in the recovery of purified M proteins with differing physical and immunological properties (10). Since the harshness of the conditions of acid extraction might account for these differences, additional methods for release of M protein which would yield a more "native" and/or homogeneous preparation (1, 9, 19) have been sought.

Of the numerous enzymatic and nonenzymatic methods that have been used for the cleavage of proteins or peptide chains, none has shown the specificity exhibited by cyanogen bromide (CnBr) (11). This chemical has the unique ability to specifically and quantitatively cleave a protein or peptide chain at the carboxyl end of methionine. Because of its specificity and the limited number of methionine residues usually found in proteins, this reagent has been particularly useful in studies of the primary structure of proteins. In our previous studies with acid-extracted and purified type 12 M protein of group A streptococci, this protein appeared to have very few methionine residues (22). Preliminary studies of acid-extracted and purified M12 protein revealed that cleavage with CnBr did not destroy the ability of M12 protein to react with type-specific antibody and suggested the possibility that CnBr might be used to extract M protein from the bacterial cell.

The present study describes a new method for the release of M protein from whole cells by treatment with CnBr. The subsequent purification and characterization of some of the physical, chemical, and immunological properties of purified type 12 M protein obtained by this method are reported.

MATERIALS AND METHODS

Organisms. Five strains of group A, type 12 M were used in these studies and were isolated originally from throat swabs or tonsilar tissue obtained from patients. The strains were stored in small portions of brain heart infusion broth at −20°C and were thawed when needed to produce starter cultures for batch growth. Cells for extraction were prepared by stationary growth in flasks containing 8 liters of brain heart infusion broth. After 16 to 18 h of growth at 35 to 37°C, the bacteria were harvested from 96 liters by sedimentation in a refrigerated centrifuge with a continuous-flow attachment. The sediment was dried by lyophilization and extracted as described below.

Extraction with CnBr. The lyophilized bacteria were suspended in phosphate-buffered saline pH 7.3 (PBS) at a ratio of 10 ml of PBS/g of bacteria. The mixture was stirred at 5°C for 5 h and then centrifuged

for 15 min at a relative centrifugal force (RCF) of 22,000 in a refrigerated centrifuge. The supernatant fluid was separated and saved. The sediment was suspended in PBS to the original volume, mixed well, and centrifuged for 15 min at an RCF of 22,000. The supernatant fluid was recovered and pooled with the first and hereafter is referred to as the presoak extract.

The sediment was resuspended in 10 ml of 70% formic acid in distilled water per original g of lyophilized bacteria. A 3-g portion of CnBr crystals (Eastman Kodak, Rochester, N.Y.) was added per g of bacteria, and the mixture was stirred in a glass-stoppered tube at room temperature in a chemical hood for 18 h. The mixture was centrifuged for 15 min at an RCF of 22,000. The supernatant fluid was saved and the sediment was suspended to its original volume with 70% formic acid. After careful mixing, the suspension was centrifuged, and the resulting supernatant fluid was separated and pooled with the previous one. The sediment was discarded.

The pooled supernatant fluids (100 to 200 ml) obtained by extraction with CnBr were dialyzed for 18 h in Visking tubing at 4°C against six changes of 6,000 ml of PBS. The dialyzed mixture was centrifuged for 15 min at an RCF of 22,000, and the supernatant fluid was saved and is hereafter referred to as CnBr extract.

Hydroxylapatite column chromatography. The bacterial extracts were separated by column chromatography with hydroxylapatite as described previously (13). In brief, before separation, the extracts were dialyzed against the starting buffer, 0.01 M sodium phosphate (pH 6.7). Then 20 to 35 mg of protein was layered onto a column of hydroxylapatite (3.0 by 2.0 cm) in the starting buffer. After the sample was applied, the column was washed with the 0.01 M phosphate buffer until the absorbance (280 nm) of the eluate had returned to the base line. Stepwise elution of the adsorbed proteins was performed with 0.1 and 0.3 M sodium phosphate buffer (pH 6.7). The tubes containing protein were combined to form three pools of the proteins eluted by the 0.01, 0.1, and 0.3 M buffers. The pools from six to eight column runs were combined, dialyzed for 24 h at 4°C against the 0.1 M ammonium carbonate (pH 8.3), and lyophilized. The dry powders were stored at 4°C in a desiccator over P_2O_5.

Antisera. Absorbed, type 12 M-specific and group A antisera were kindly provided by the Bureau of Laboratories, Center for Disease Control, Atlanta, Ga. Antibodies to the column fractions were raised in randomly bred New Zealand white rabbits by a single subcutaneous injection of approximately 320 μg of protein nitrogen emulsified in complete Freund adjuvant. Antibodies to whole cell vaccines of group A, types 1, 3, 6, 12, and 17 M were raised in rabbits by repeated intravenous injections. An unabsorbed antiserum for streptococcal teichoic acid (M49, E4 pool D, 4/20/72) was kindly provided by W. K. Harrell, Center for Disease Control, Atlanta, Ga. This serum was rich in antibodies for polyglycerophosphate and E4 when tested by immunoelectrophoresis (23).

Immunological techniques. Double diffusion in 1% Noble agar and immunoelectrophoresis, using 1% Noble agar in barbital buffer, pH 8.2 (ionic strenght [μ], 0.05), were performed as described previously (22). Streptococcal T antigen was assayed by an agglutination-inhibition system using trypsin-treated T12 bacteria and the standard CDC T12 agglutinating antiserum (12). Opsonic antibodies were measured by Richard Facklam, Center for Disease Control, Atlanta, Ga., by the indirect bactericidal test essentially as described by Lancefield (15).

Physical-chemical determinations. Polyacrylamide disc gel electrophoresis was performed by a modification of the method of Ornstein and Davis (13). Molecular weights were determined by electrophoresis in 13 and 15% polyacrylamide disc gels with sodium dodecyl sulfate (SDS) and a discontinuous buffer system (18). The samples were treated with 2% SDS and 2% 2-mercaptoethanol for 3 h at 37°C as originally suggested (20). Molecular weights were estimated by comparison of the mobilities of the M proteins with those of whale myoglobin, trypsin, pepsin, bovine plasma albumin, ovalbumin, lysozyme, and chymotrypsin.

Protein concentrations were determined by the method of Lowry et al. (17), using bovine albumin as a reference standard, and expressed as protein nitrogen. Methylpentoses were measured by the method of Dische and Shettles (7).

Amino acid composition was determined on an automatic Beckman model 120C amino acid analyzer with a single-column technique utilizing sequential elution with four sodium citrate solutions (6). Samples were hydrolyzed for 24 h at 110°C in constant boiling HCl under a nitrogen atmosphere.

N-terminal amino acids were identified by the dinitrophenylation method essentially as described by Brenner et al. (3). The dinitrophenylamino acids of M protein were determined with one- and two-dimensional, ascending thin-layer chromatography by comparison with the mobilities of known dinitrophenylamino acids in various solvents. Separation was attained with chloroform–benzyl alcohol–glacial acetic acid (70 + 30 + 3), benzene–pyridine–glacial acetic acid (80 + 20 + 2) or toluene–pyridine–ethylene chlorohydrin–0.8 N ammonium solution (100 + 30 + 60 + 60). Lysine could not be distinguished from tyrosine under these conditions of chromatography.

RESULTS

Conditions for extraction. Several aspects of the conditions for extraction of cellular antigens by digestion with CnBr were assessed. After lyophilization, standard dry weights of cells were either soaked in PBS, 70% formic acid, or nothing for 5 h before they were extracted with CnBr in 70% formic acid. The presoak extracts with PBS and 70% formic acid were only weakly reactive with group A and type 12-specific antisera and contained other cross-reactive antigens. The subsequent CnBr extracts of these presoaked cells gave strong reactions with group A and type 12-specific antisera and an unabsorbed antiserum to type 6 M; however, the extracts obtained from cells which were not presoaked gave strong reactions with group A antiserum

but only weak reactions with type 12-specific antiserum. A portion of the heavy precipitate, which formed in the reaction using lyophilized cells which had not been presoaked, was soluble in PBS and gave a strong reaction with type 12-specific antiserum. Because of the above differences, we decided to routinely presoak the lyophilized cells in PBS before extraction to maintain M protein in the soluble state. Extraction of the cells in 70% formic acid without the addition of CnBr did not release M protein from the cells.

With the conditions described above, a second extraction with CnBr did not yield additional proteins that reacted with type 12 antiserum, although substances reactive with group A antiserum were still released. Acid extraction by the method of Lancefield (14) of cells previously treated with CnBr did not yield additional proteins which reacted with type 12-specific antiserum and suggested that M protein was completely released by CnBr. Cells subjected to a single extraction with CnBr retained their gross morphology and their ability to stain gram positive when examined by light microscopy.

Amounts of protein released. The amounts of protein released from three strains of group A, type 12 M streptococci as a result of presoaking the lyophilized cells in PBS and by extraction of the hydrated cells with CnBr are shown in Table 1. Approximately two to three times as much protein was released by extraction with CnBr as was found in the presoak fluid. The proteins released by CnBr represented approximately 5% of the dry weight of the cells.

Hydroxylapatite column chromatography. The extracted proteins could be separated further by elution from columns of hydroxylapatite (Table 1). Eighty to 100% of the protein applied to the column was recovered. The proportions of protein recovered in the wash-through fraction (0.01 M) and in the 0.1 and 0.3 M eluates differed for the presoak and CnBr extracts, with a greater proportion of the proteins in the latter not adsorbing to the columns. The profiles of the eluted proteins were similar for each of the three strains of type 12 M streptococci analyzed in this manner. The 0.3 M eluates of the CnBr extracts contained nearly all of the proteins reactive with type 12-specific antiserum, and in each instance these proteins represented approximately 1% of the dry weight of the cells.

Antigenic analysis. The reactivity of the hydroxylapatite column fractions of four strains of type 12 M streptococci with various antisera was assessed. The 0.3 M fractions of the CnBr extract for each strain contained all or nearly all of the proteins reacting with type 12-specific antiserum. These same fractions also contained a non-type-specific antigen which reacted with an unabsorbed antiserum prepared against whole cells of a strain of group A, type 6 M, but not with those raised against strains of types 1, 3, and 17 M (Fig. 1). The presoak, 0.3 M column fractions appeared to contain one or more antigens which reacted with the unabsorbed antisera from types 1, 3, 6, and 17 M.

T12 antigen was not detected in the 0.01, 0.1, and 0.3 M CnBr eluates by either double diffusion in agar using the standard CDC agglutinating T12 antiserum reconstituted in 1 ml rather than 2 ml of distilled water or the agglutination-inhibition system. The 0.1 and 0.3 M column fractions of the CnBr extracts of strains 3 and 4 were tested for their reactivity with an unabsorbed antiserum rich in antibodies for streptococcal glyceroteichoic acid (Fig. 2). A strong reaction with known streptococcal teichoic acid (obtained from Edward Beachey, Veterans Ad-

TABLE 1. *Amounts of protein in presoak and CnBr extracts of whole cells and in fractions of these extracts obtained by column chromatography with hydroxylapatite*

Strain	Whole cells				Column chromatography (%)				
	Bacteria (g [dry wt])	Protein presoak (mg)	Protein CnBr extracted (mg)	Dry wt (%)	Protein recovered	0.01 M	0.1 M	0.3 M	0.3 M as % of bacteria dry wt
4	4	95		2	80	27	56	17	0.3
			191	5	100	50	27	23	1.2
5	4	122		3	100	26	59	15	0.5
			231	6	97	53	26	21	1.2
6	4	68		2	95	30	53	17	0.3
			211	5	83	57	26	17	0.8

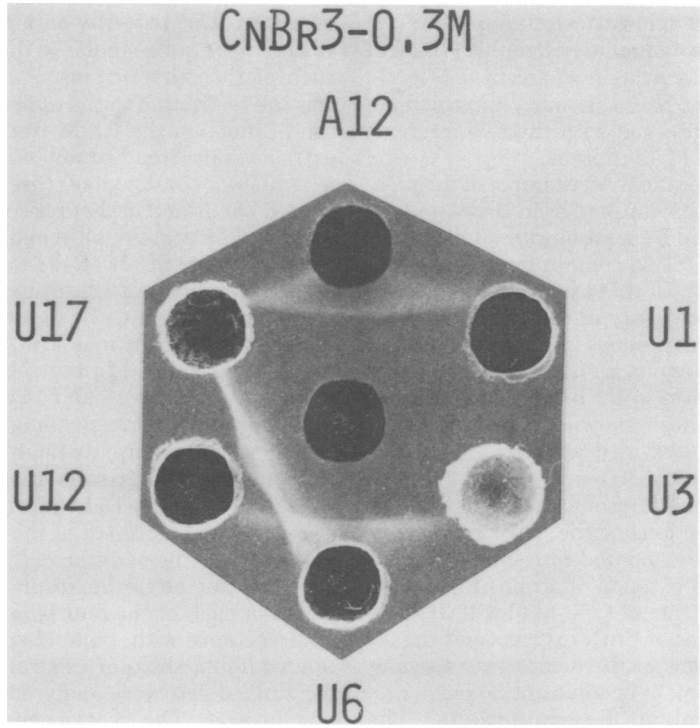

FIG. 1. *Double diffusion in agar of the 0.3 M hydroxylapatite column eluate of CnBr 3 (192 μg of protein nitrogen per ml) against type-specific antiserum (A12) and unabsorbed antisera (U1, 3, 6, 12, and 17).*

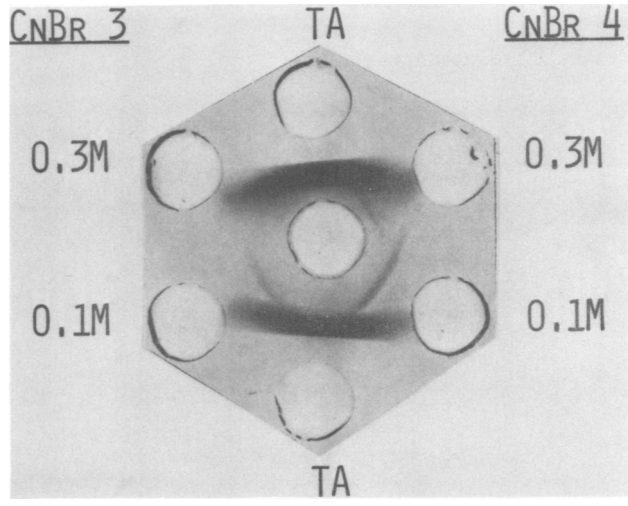

FIG. 2. *Reactions in double diffusion in agar of the 0.1 and 0.3 M eluates of CnBr 3 (192 and 258 μg of protein nitrogen per ml) and CnBr 4 (382 and 332 μg of protein nitrogen per ml) and of streptococcal teichoic acid (TA) with an unabsorbed rabbit antiserum raised to streptococcal glyceroteichoic acid (center well).*

ministration Hospital, Memphis, Tenn.) was obtained, but a similar reaction was not observed with the CnBr fractions. This serum contained precipitating antibodies for at least two other antigens which were not further defined; one of these antigens was present in both 0.1 M fractions, and the other was present only in the 0.3 M fraction of strain 4.

The mobility of acid-extracted M protein (22) was compared by immunoelectrophoresis with CnBr-extracted M protein of strain 5 (Fig. 3). The CnBr extract had a mobility intermediate between those observed with the two fractions of acid-extracted M 12 protein.

Opsonic antibodies. An example of the measurement of opsonic antibodies in the 8- and 14-week sera obtained from rabbits immunized with either the 0.3 or 0.1 M column fractions is presented in Table 2. In this example, opsonic antibodies are present only in the 8- and 14-week sera obtained from animals immunized with the 0.3 M column fractions. In other measurements opsonic antibodies, with bactericidal indexes comparable to those shown in Table 2, were detected in sera obtained as early as 4 weeks after immunization.

The stimulation of opsonic antibodies by the three-column fractions of the CnBr extracts of strains 3 and 4 are compared with similar column fractions for acid extracts of strains 3 and 4 as well as another strain of type 12 (Table 3). Only the 0.3 M fractions of CnBr extracts and the 0.3 and 0.1 M fractions of the acid extracts stimulated the formation of opsonic antibodies.

Physical-chemical determinations. The proteins present in column fractions of the presoak and CnBr extracts were examined by electrophoresis in heavily loaded polyacrylamide disc gels. The patterns obtained with strain 3 (Fig. 4) were quite similar to those obtained with each of the other strains. Particularly striking was the restriction to the upper 12% of the gel of the proteins in the 0.3 M fraction of the CnBr extracts. The broad zone obtained with the 0.3 M fraction consisted of three to five closely spaced bands and in that regard resembled acid-extracted M protein, although the mobilities of the latter were faster (R_f 23 to 32%) (Fig. 5).

The mobilities of the proteins in the 0.3 M column fraction of CnBr in an SDS-acrylamide gel are also shown in Fig. 5. When 100 μg of protein was applied to the gels, multiple determinations on both 13 and 15% gels revealed at least four bands corresponding to estimated molecular weights of approximately 12,000, 14,000, 18,000, and 23,000. In some preparations, a band with a molecular weight of around 35,000 was also present. Treatment of the proteins with 2% SDS without the addition of 2% 2-mercaptoethanol did not alter the result. Proteins in the region of each of the four bands formed precipitin reactions with type 12-specific antiserum when 2.5-mm slices of gels run in parallel with the stained gels were analyzed by double diffusion in agar. The type-specific immunological reaction could be readily distinguished from the nonspecific reaction of serum proteins with SDS since the latter disappeared after washing the

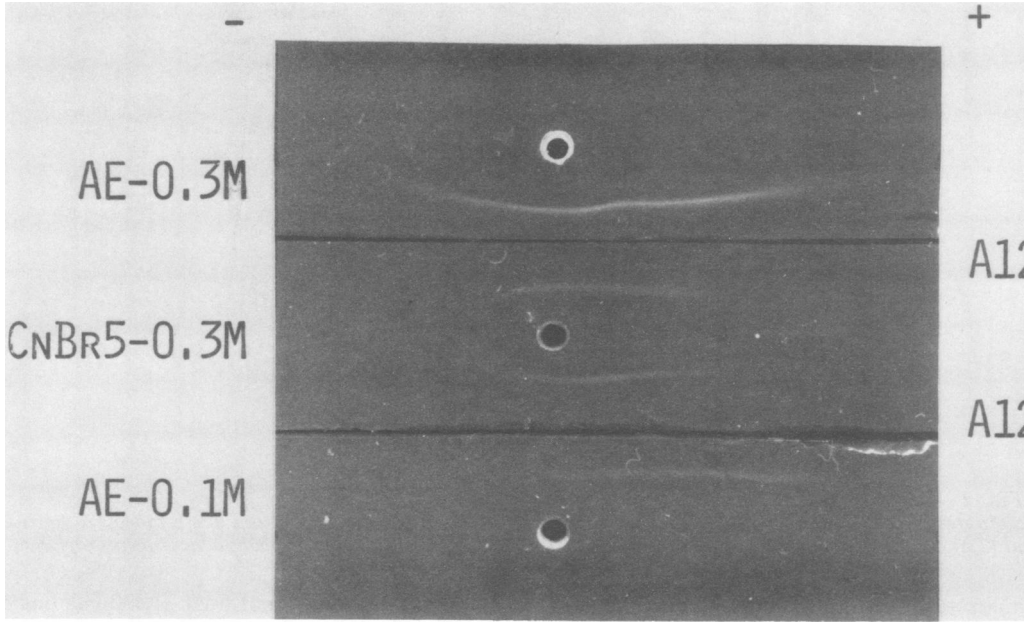

FIG. 3. *Comparison of the immunoelectrophoretic mobility of the 0.3 M hydroxylapatite column fraction of CnBr 5 and the 0.1 and 0.3 M hydroxylapatite column fractions of acid-extracted (AE) type 12M protein. Troughs contained type 12-specific antiserum (A12).*

TABLE 2. *Measurement of M12 opsonic antibodies in sera from rabbits immunized with either the 0.3 or 0.1 M column fractions of proteins extracted by CnBr3*[a]

Serum	CFU[b]			Bactericidal index[d]
	216[c]	44[c]	17[c]	
Normal rabbit	1,408	428	224	
Rabbit K36, 0.3 M fraction				
8 week	100	22	10	16
14 week	80	21	9	19
Rabbit K37, 0.3 M fraction				
8 week	188	69	38	7
14 week	300	82	48	5
Rabbit K34, 0.1 M fraction				
8 week	1,056	344	172	1
14 week	960	644	120	1
Rabbit K35, 0.1 M fraction				
8 week	864	300	264	1
14 week	1,416	400	120	1

[a] Performed by R. R. Facklam, Center for Disease Control, Atlanta, Ga.
[b] Colony-forming units after 2 h of incubation with serum.
[c] Zero time inoculum.
[d] Bactericidal index = sum of counts all challenge concentrations for normal rabbit serum at 2 h/sum of counts all challenge concentrations for test serum at 2 h.

plates for 48 h with PBS.

Methylpentoses were not detected in a 1-ml sample of the 0.3 M column fraction of CnBr 3, which contained 650 μg of protein.

The compositions of amino acids in the 0.1 and 0.3 M column fractions obtained from CnBr extracts of three different strains are compared in Table 4. Analyses of the same column fractions were quite similar for the three strains. Although glutamic acid, lysine, alanine, aspartic acid, leucine, and glycine were the six most common amino acids in each of the fractions tested, the relative proportions of a number of the amino acids differed when the 0.3 M fractions were compared with 0.1 M fractions. Methionine was not present in any of the fractions, confirming the completeness of the reaction with CnBr. Approximately 2 residues of homoserine per 1,000 were found in the 0.3 M fractions as compared with 13 to 15 in the 0.1 M fractions.

The 0.3 M fractions of strains 3 and 4 appeared to have N-terminal alanine and either lysine or tyrosine or both when examined by ascending two-dimensional chromatography with chloroform–benzyl alcohol–glacial acetic acid (70 + 30 + 3) in the first dimension and toluene–pyridine–ethylene chlorhydrin–0.8 N ammonium solution (100 + 30 + 60 + 60) in the second. Under these conditions it was not possible to differentiate lysine from tyrosine.

DISCUSSION

Although CnBr has been used primarily in studies of the structure of pure proteins or peptide chains, the present work demonstrates the feasibility of using this reagent to release streptococcal, type 12 M protein from whole cells. The released proteins accounted for approximately 5% of the dry weight of the bacteria. Since CnBr acts by cleaving proteins specifically at the carboxyl end of methionine, the release of proteins or their peptide fragments implies that they have one or more exposed methionines capable of reacting with this reagent. The completeness of the reaction was confirmed by the absence of methionine and the presence of homoserine or homoserine lactone in the amino acid analyses of the released proteins and by the failure of acid extraction by the method of Lancefield to yield additional M protein from cells previously treated with CnBr. Purified M protein represented approximately 1% of the dry weight of the bacteria. The T antigen of type 12 streptococci did not appear to be released by CnBr, suggesting inaccessibility or destruction of this antigen by CnBr. In preliminary experiments, we have been able to extract M proteins from the whole cells of streptococcal M types 6 and 17. Thus CnBr provides an additional, highly specific method for the release of streptococcal M proteins, which should prove useful in the analyses of structural-functional relationships among different M proteins.

The recovery from CnBr extracts of proteins which retained the immunological and biological criteria of type 12 M protein demonstrated that methionine was not essential to the structural arrangements of the proteins which account for these important properties. These proteins, which formed precipitins with type-specific antibodies and which stimulated the formation of opsonic antibodies in rabbits, could be separated from other nonreactive proteins by column chro-

TABLE 3. *Stimulation of M12 opsonic antibodies by hydroxylapatite column fractions of proteins extracted by acid or CnBr*

Extract	Strain	Column fraction (M)		
		0.3	0.1	0.01
Acid	12S	1/1[a]	1/1	0/1
	12B	3/3	3/3	0/1
	12-126/4	1/1	1/1	0/1
CnBr	3 (12S)	2/2	0/2	0/2
	4 (126/4)	2/2	0/2	0/1

[a] Number of rabbits forming opsonic antibodies per number immunized.

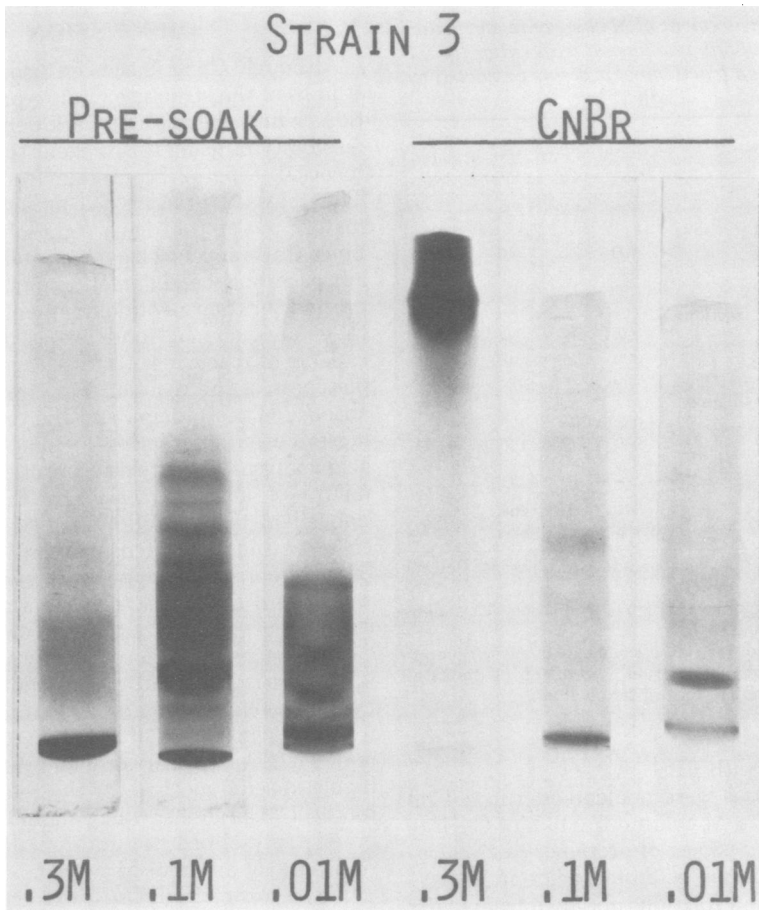

FIG. 4. *Distribution of proteins following electrophoresis in standard polyacrylamide disc gels of the presoak and CnBr hydroxylapatite column fractions of strain 3.*

matography with hydroxylapatite. The purified fraction of type 12 M protein was free of T antigen and teichoic acid but included a single non-type-specific antigen. The association of cross-reactive antigens with M proteins has been encountered commonly among purified M proteins obtained by nearly all methods used for their extraction and purification (1, 4, 8, 10, 21, 22). These cross-reactive antigens have been shown to coprecipitate with M protein and presumably could represent antigenic determinants within M protein rather than a contaminating antigen (21). However, at least two preparations of different types of M protein, which were free of cross-reactive antigens (2, 22), have been recovered. These findings demonstrated that even if the cross-reactive antigens represented determinants within the native molecules of these two types of M protein, their location was not critical to the expression of those determinants which reacted with type-specific precipitins and stimulated opsonic antibodies. In addition, others have found that the determinants responsible for reacting with type-specific precipitating and opsonic antibodies were different and may be separated. Thus Russell and Facklam (19) and Fischetti et al. (9), recovered fractions of types 6 and 12 M protein which reacted with precipitating, but not with opsonic, antibodies. On the other hand, Cunningham and Beachey (5) isolated a fraction of type 24 M protein which reacted with opsonic but not with precipitating antibodies and which was not immunogenic. The CnBr extracts in the present study may have contained similar fragments of M protein which were not separated and identified by the methods used for the purification and characterization of the M protein. Although each of the 3 to 5 protein bands present in our final purified fraction appeared capable of reacting with precipitating antibodies, a fragment of M protein, which could react with precipitating antibodies

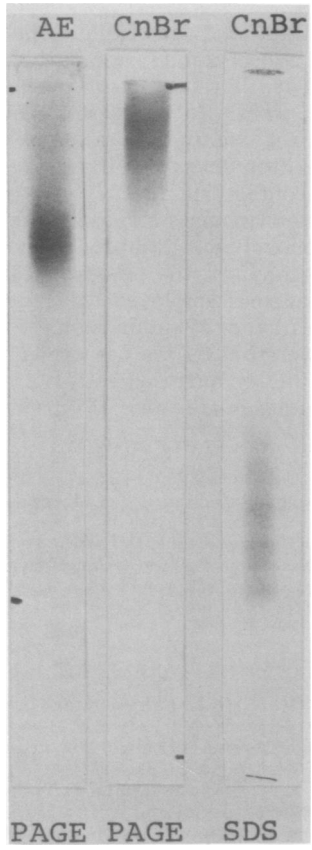

FIG. 5. *Distribution of protein bands in the 0.3 M eluate of acid (AE) or CnBr-extracted M protein on electrophoresis in the standard polyacrylamide disc gel (PAGE) or in a 13% SDS-PAGE system.*

but was not able to stimulate the formation of opsonic antibodies, would have been missed since only the whole fraction was used to immunize rabbits. Similarly, since the ability of the various fractions to inhibit opsonization was not assessed, a fragment of M protein, which neither reacted with precipitating antibodies nor stimulated the formation of opsonic antibodies, may have gone unrecognized in those column fractions.

The physical-chemical characteristics of the CnBr-extracted and purified fraction of M protein were similar to those previously derived by other methods. The final preparation was free of contamination with other cell wall components. On electrophoresis in a standard polyacrylamide disc gel system, the type 12 M proteins recovered from CnBr extracts moved as 3 to 5 closely spaced bands with R_f's of less than 12%. Electrophoresis in the SDS-polyacrylamide system revealed four bands with estimated molecular weights of 12,000, 14,000, 18,000 and 23,000. Multiple protein bands have been noted previously with type 12 M proteins released by extraction with acid hydrolysis (13, 22) or guanidine hydrochloride (19); however, the R_f's of such bands in a standard system for polyacrylamide gel electrophoresis have generally ranged from 20 to 60%. The estimated molecular weight was 32,000 for the smallest fraction recovered from acid extracts which still possessed all the biological characteristics of M protein (22). However, an additional minor protein band was present at the dye front. In the previously employed system for SDS-polyacrylamide gel electrophoresis, proteins moving at the dye front would have had molecular weights of 17,000 or less, and thus this band may have consisted of an array of molecular sizes similar to those found for CnBr-extracted type 12 M protein with the present system for SDS-polyacrylamide gel electrophoresis. Similar results have been reported previously for type 6 M protein released by a nonionic detergent (8). These studies have provided the most definitive support to date for the concept that the multiple protein bands represent multiple molecular forms of M protein with a subunit structure that may be 6,000 daltons or less.

The total amino acid composition of the CnBr-extracted and -purified type 12 M proteins of three different strains was similar to that noted previously with two purified fractions of acid-extracted type 12 M protein (22) and to those described for other M types (10). Generally, the reported amino acid determinations have reflected a composite of the multiple protein bands present in the purified preparations and thus may have masked differences in the individual protein bands. However, Fischetti et

TABLE 4. *Amino acid analysis of column fractions of CnBr extracts of three strains of type 12 M*

Amino acid	CnBr 3 (M)		CnBr 4 (M)		CnBr 6 (M)	
	0.3	0.1	0.3	0.1	0.3	0.1
Asp	101.6[a]	116.4	104.5	112.3	103.9	109.7
Thr	35.7	60.6	30.9	55.7	32.6	52.4
Ser	46.3	48.3	42.1	50.6	46.5	48.9
Glu	183.5	133.9	190.7	130.4	185.8	137.3
Pro	5.9	15.8	4.4	18.1	8.7	12.6
Gly	59.6	91.1	62.2	95.0	58.4	95.7
Ala	142.7	110.9	139.3	105.6	143.4	118.5
Cys/2	0.6	0.7			0.4	
Val	44.5	65.9	44.3	64.2	41.5	65.1
Ileu	29.7	50.2	27.1	47.9	28.2	65.1
Leu	100.5	79.7	108.1	80.8	100.6	79.1
Tyr	11.1	20.7	12.7	22.2	10.0	17.7
Phe	15.8	24.0	14.6	24.9	14.3	23.4
Lys	148.9	100.1	147.7	105.5	154.5	114.8
His	15.1	18.5	14.8	19.0	14.6	15.2
Arg	56.2	50.3	54.4	53.2	55.2	47.0
HSer	2.4	12.9	2.2	14.5	1.5	15.0

[a] Residues per 1,000.

al. (9) found that the amino acid compositions were similar for the groups of three to four bands of type 6 M protein with molecular weights ranging from 6,000 to 35,000. The occurrence of at least two N-terminal amino acids—alanine and either lysine or tyrosine or both—in CnBr-extracted and purified type 12 M protein contrasts with our previous finding of only alanine for acid-extracted type 12 M protein (22). Variation in the N-terminal amino acids for fragments of M proteins released by different methods of extraction would not be unexpected since the molecules may be cleaved at different sites. The reason for the occurrence of two or possibly three N-terminal amino acids in the purified fraction of CnBr-extracted M protein is not clear at this time but may reflect the presence of contaminating proteins, or indicate that M protein released by CnBr consists of two or more different, closely associated or cross-linked, peptide chains. Unfortunately, N-terminal analyses of the various molecular weight fractions of type 6 M protein recovered by Fischetti et al. (9) were not reported. Although the total amino acid compositions of each of their fractions which contained three to four protein bands were similar, this does not exclude the possibility that each of these fractions contained more than a single, different peptide chain. Recent work by Beachey et al. provides the strongest support for M protein consisting of only a single peptide chain (2). A fragment of type 24 M protein was recovered from a pepsin extract of whole cells by purification with differential fractionation with ammonium sulfate, column chromatography, and isoelectric focusing. This purified fragment was free of cross-reactive determinants, but it possessed the antigenic determinants capable of reacting with type-specific precipitating and opsonic antibodies and of stimulating the formation of opsonic antibodies in rabbits. In addition, this purified fragment formed a single band in SDS-polyacrylamide gel electrophoresis with a molecular weight of 33,500 and possessed N-terminal valine. Since only a single amino acid was released at each step in the sequencing of the first 29 amino acids, the protein apeared to consist of only a single peptide chain rather than two, closely linked, different chains with the same N-terminal amino acid.

Finally, it cannot be emphasized too strongly that in comparing the findings in the present and previously reported studies one must keep in mind that the different methods of extraction and purification may account for some of the reported differences in the characteristics of M proteins. Thus, M protein almost certainly consists of a range of protein fragments when released by partial acid or enzymatic hydrolysis, alkaline conditions, denaturing agents such as guanidine hydrochloride, nonionic detergents, and the currently reported cleavage of methionine residues with CnBr. These fragments may include intact, "native" M protein as well as pieces from different sites of the native structure and may contain all, or only a part, of the antigenic determinants responsible for the immunological and biological properties of M protein. Unfortunately, the effects of each of the different schemes employed for the extraction and purification of M proteins on the results of their characterization are not known since the different schemes have generally not been used to study strains of the same M type.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI 6964 from the National Institute of Allergy and Infectious Diseases.

We express our appreciation to Pamela Thomas and W. Joseph Kowalski for their expert technical help, to Isabelle Smith for her assistance in the preparation of this manuscript, and to John Daniels for performance of the total amino acid analyses.

LITERATURE CITED

1. **Beachey, E. H., G. L. Campbell, and I. Ofek.** 1974. Peptide digestion of streptococcal M protein. II. Extraction of M antigen from group A streptococci with pepsin. Infect. Immun. **9**:891–896.
2. **Beachey, E. H., G. H. Stollerman, E. Y. Chiang, T. M. Chiang, J. M. Seyer, and A. H. King.** 1977. Purification and properties of M protein extracted from group A streptococci with pepsin: covalent structure of the amino terminal region of type 24 M antigen. J. Exp. Med. **145**:1469–1483.
3. **Brenner, M., A. Niederwieser, and G. Pataki.** 1965. Amino acids and derivatives, p. 414–427. *In* E. Stahl (ed.), Thin-layer chromatography, a laboratory handbook. Academic Press, Inc., New York.
4. **Cunningham, M. W., and E. H. Beachey.** 1974. Peptic digestion of streptococcal M protein. I. Effect of digestion of suboptimal pH upon the biological and immunochemical properties of purified M protein extracts. Infect. Immun. **9**:244–248.
5. **Cunningham, M., and E. H. Beachey.** 1975. Immunochemical properties of streptococcal M protein purified by isoelectric focusing. J. Immunol. **115**:1002–1006.
6. **Daniels, J. R., and G. H. Chu.** 1975. Basement membrane collagen of renal glomerulus. J. Biol. Chem. **250**:3531–3537.
7. **Dische, Z., and C. B. Shettles.** 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
8. **Fischetti, V. A.** 1977. Streptococcal M protein extracted by nonionic detergent. II. Analysis of the antibody response to multiple antigenic determinants of the M protein molecule. J. Exp. Med. **146**:1108–1123.
9. **Fischetti, V. A., E. C. Gotschlich, G. M. Siviglia, and J. B. Zabriskie.** 1976. Streptococcal M protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type-specific molecules. J. Exp. Med. **144**:32–53.
10. **Fox, E. N.** 1974. M proteins of group A streptococci. Bacteriol. Rev. **38**:57–86.
11. **Gross, E.** 1967. The cyanogen bromide reaction. Methods Enzymol. **11**:238–255.

12. **Johnson, R.** 1975. Characterization of group A streptococcal R-28 antigen purified by hydroxyapatite column chromatography. Infect. Immun. **12**:901-909.
13. **Johnson, R. H., and K. L. Vosti.** 1968. The purification of two fragments of M protein from a strain of group A type 12 streptococcus. J. Immunol. **101**:381-391.
14. **Lancefield, R. C.** 1928. The antigenic complex of *Streptococcus hemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus hemolyticus*. J. Exp. Med. **47**:91-103.
15. **Lancefield, R. C.** 1957. Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. J. Exp. Med. **106**:525-544.
16. **Lancefield, R. C.** 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89**:307-313.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
18. **Maizel, J. V., Jr.** 1971. Polyacrylamide gel electrophoresis of viral proteins, p. 179-246. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press, Inc., New York.
19. **Russell, H., and R. R. Facklam.** 1975. Guanidine extraction of streptococcal M protein. Infect. Immun. **12**:679-686.
20. **Shapiro, A. L., E. Viñuela, and J. V. Maizel.** 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. **28**:815-820.
21. **Vosti, K. L.** 1975. Characterization of a non-type-specific antigen(s) associated with group A streptococcal type 12 M protein. Infect. Immun. **11**:1300-1305.
22. **Vosti, K. L., R. H. Johnson, and M. F. Dillon.** 1971. Further characterization of purified fractions of M protein from a strain of group A, type 12 streptococcus. J. Immunol. **107**:104-114.
23. **Wilson, A. T., and G. G. Wiley.** 1963. The cellular antigens of group A streptococci. Immunoelectrophoretic studies of the C, M, T, PGP, E_4, F and E antigens of serotype 17 streptococci. J. Exp. Med. **118**:527-556.